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Carbohydrate and elicitor enhanced withanolide (withaferin A and withanolide A) accumulation in hairy root cultures of *Withania somnifera* (L.)

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Leaves of *Withania somnifera* contained more withaferin A and withanolide A than roots indicating that these compounds mainly accumulate in leaves. With an increase in age of the plant, withaferin A was enhanced with a corresponding decrease in withanolide A. Hairy root cultures were induced from leaf explants using *Agrobacterium rhizogenes* and the transgenic nature of hairy roots was confirmed by partial isolation and sequencing of *rol*B gene, which could not be amplified in untransformed plant parts. In hairy roots, withaferin A accumulated at 2, 3 and 4% but not at 6% sucrose, the highest amount being 1733 µg/g dry weight at 4% level. High and equal amounts of withaferin A and withanolide A accumulated (890 and 886 µg/g dry tissue respectively) only at 3% sucrose. Increasing concentrations of glucose enhanced withaferin A and it peaked at 5% level (3866 µg/g dry tissue). This amount is 2842 and 34% higher compared to untransformed roots and leaves (collected from 210-day-old plants) respectively. Withanolide A was detected at 5% glucose but not at other concentrations. While chitosan and nitric oxide increased withaferin A, jasmonic acid decreased it. Acetyl salicylic acid stimulated accumulation of both withaferin A and withanolide A at higher concentrations. Triadimefon, a fungicide, enhanced withaferin A by 1626 and 3061% (not detected earlier) compared to hairy and intact roots respectively.

Keywords: Elicitors, Hairy roots, *Withania somnifera*, Withasteroids

Withania somnifera (L.) (family Solanaceae) is an important medicinal plant that produces steroidal lactones (withanolides) and saponins as withanolide glucoconjugates. The plant has antitumor, antistressor, immunomodulatory, antiinflammatory, radio-sensitizing and antibacterial activities[1]. Roots contain steroidal compounds including ergostane type steroidal lactones like withaferin A, withanolides A-Y, withasomniferin A, withasomidicnone, withasomniferols A-C, withanone, a few flavonoids and reducing sugars[2]. But, low yields from the natural sources, large genotypic and chemotypic variations[3], severe insect attacks during active growth, long gestation period between planting and harvesting are some of the constraints for the large scale harvesting and production of withanolides. In this regard, hairy root cultures have been thought as comparatively fast growing and genetically more stable. Accumulation of no or low content of withanolides was reported from untransformed cultures of *W. somnifera*[4]. On the other hand, the results of Sharada *et al*.[5] and Bandyopadhyay *et al*.[6] suggested that production of withanolides is closely associated with morphological differentiation. However, there are no reports on the effect of carbohydrates or elicitors such as chitosan, jasmonic acid (JA), acetyl salicylic acid (ASA) and signaling molecules such as nitric oxide on the accumulation of withanolides in hairy roots of *W. somnifera*. This communication reports accumulation of withanolides in roots, shoots and leaves collected from untransformed plants and also enhancement in withaferin A and withanolide A, in stably transformed hairy roots of *W. somnifera* as influenced by different carbohydrates, elicitors and a fungicide.

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Materials and Methods

Explant source—Seeds of *Withania somnifera* (L.) Dunal (cultivar Poshitha, not an elite species), 4 and 7-month-old plant material were procured from the

Central Institute of Medicinal and Aromatic Plants, Hyderabad. Young leaf explants measuring 2-3 cm were excised from *in vitro* germinated seedlings. The explants were pricked with sterile hypodermic needles that were dipped in a 48 h old suspension culture of *Agrobacterium rhizogenes* strain 15834, grown in yeast, mannitol broth[7] medium. The infected leaf explants were kept under dark for 2 days in Murashige and Skoog's basal medium[8] solidified with 0.3% gelrite. After cocultivation of explants with *Agrobacterium* for two days, the explants were rinsed thrice with sterile distilled water, and transferred to MS basal medium supplemented with 2% sucrose, 500 mg/L carbenicillin and 500 mg/L cefotaxime as the decontaminating antibiotic and incubated under dark at 25 °C. The axenic hairy roots were cut and subcultured in hormone free 40 mL of MS medium containing 2% sucrose and incubated at 25±1 °C in a culture room under 12 h light (40 µmol m^{-2} s^{-1}) /dark cycles. Chitosan, JA, ASA, sodium nitroprusside (a compound that releases nitric oxide) and triadimefon [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone] (Bayer), a fungicide were added as elicitors into the medium by filter sterilization.

Growth measurements—Hairy roots grown for 25 days were taken from the culture vessels and fresh weights (FW) were measured on a pre-weighed aluminium foil. Dry weights (DW) of the roots were measured after shade drying them at room temperature for 4 days.

Confirmation of hairy roots by rolB sequencing—DNA was isolated from roots, leaves of untransformed plants and from hairy roots and quantified. Genomic DNA isolated from roots and leaves of untransformed plants was used as negative control. Primers spanning *rol*B gene were used in the PCR reaction to detect T_L T-DNA (5′-CCTGCTTTCCAGAAACGAT-3′, and 5′GAGTCGCAGGGTTAGGTCTG-3′), the expected fragment size being 536 base pairs. The amplicon was eluted directly from the gel and sequenced (Bioserve, Hyderabad). The partial nucleotide sequence was then searched for homology using BLAST search tool. The nucleotide sequence of *rol*B gene isolated from *A. rhizogenes* strain 15834 was submitted to the NCBI (Accession Number EF589671). Plasmid DNA was isolated using standard alkali lysis protocol. PCR based molecular analysis of hairy roots was carried out for the transgenic nature using *rol*B gene specific primers. DNA from hairy roots was extracted and electrophoresed in 1% agarose gel prepared in 1X TAE buffer for 3 h at 60V and was quantified. Approximately 50 ng of DNA sample was used for each PCR reaction. DNA was amplified in a 25 µL reaction containing 0.5 µL of 10 mM dNTP mix (Promega), 1 µL of forward and reverse primers each, 2 µL of plasmid DNA, or DNA from tissues, 2.5 µL of Taq polymerase buffer and 1 µL of Taq DNA polymerase. PCR amplification was performed in a programmable thermal cycler under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min with final extension at 72°C for 5 min. PCR amplified products were subjected to electrophoresis in 1.2% agarose gels prepared in 1X TAE for 1 h at 80V for 45 min. PCR amplification of *rol*B was noticed in hairy roots but not in roots, shoots and leaves of untransformed plants.

Analysis for withanolides by HPLC—Shade dried material (1 g) was powdered and extracted with methanol and chloroform in 1:1 ratio. The extract was filtered with a Wattmann filter paper and concentrated under normal room temperature and pressure in Amberlight bottles for 3 days. Crude, dried extract was weighed and analyzed using HPLC along with standard withaferin A and withanolide A purchased from Natural Remedies Pvt. Ltd., Bangalore, India. Following HPLC conditions were used for analysis: Schimadzu (Japan) model-LC-10-AVP-C18 with qalisil BDS column (5 µm, 4.6 mm × 250 mm), water and acetonitrile (6:4 v/v), flow rate 1 mL/min, injection volume 20 µL, sample concentration 1 mg/5 mL in acetonitrile. Retention times for withaferin A and withanolide A were 7.936 and 12.235 which were recorded at 227 and 4 nm respectively. All the experiments were repeated at least twice. The data represented for growth and withanolide analysis are mean values collected from 10 replicates from two independent experiments. Standard errors of mean were calculated and the level of significance (*P* value) was ascertained by Least Significant Difference method.

Results

Detection of withanolides in intact plant parts—While increase in age of the plants increased withaferin A irrespective of the plant part,

withanolide A decreased in both roots and leaves (Table 1). Leaves from 120-day-old plants accumulated more withanolide A (823 µg/g DW) than leaves from 210-day-old (586 µg/g DW) plants (Table 1).

Induction of hairy roots and effect of carbohydrates on withanolide accumulation—Leaf explants were co-cultivated with *A. rhizogenes* for two-days. Hairy roots were induced after 3weeks of inoculation with 10% frequency upon culturing leaf explants in MS basal medium containing 2% sucrose. They were maintained on the same medium with 20-25-day subcultures. Morphological variations of hairy roots growing in presence of 3% sucrose, 2% glucose and 2% maltose were observed and shown in Fig. 1a, 1b and 1c respectively. While *rol*B gene amplification was noticed in hairy roots, it was not observed in untransformed roots and leaves (Fig. 2). Partial clone of *rol*B (536 bp) gene was isolated from T_L DNA of Ri plasmid (from hairy roots), its nucleotide sequence was determined which exactly matched the sequence of the strain, thus confirming the transgenic nature of roots. The growth of one of the selected hairy root lines (growing in MS medium containing 3% sucrose) is measured by fresh and dry weights and the amount of withaferin A and withanolide A was quantified using HPLC. The retention times for both withaferin A and withanolide A are shown in Fig. 3. The amounts of these two compounds accumulated as influenced by different concentrations of sucrose (1 to 6%), glucose (1, 2, 4, 5, 6, and 8%) and fructose (1 to 4%) and also the percent increase of withaferin A over that of control (MS basal + 2% sucrose) is shown in Table 2. Increase in the concentration of sucrose increased the hairy root biomass. While only withaferin A was detected at 2% sucrose (256 µg/g DW), almost equal amounts of withaferin A (890 µg) and withanolide A (886 µg) were noticed at 3%. Still higher concentrations of sucrose (4%) enhanced withaferin A, but decreased withanolide A (Table 2). At 6% sucrose, hairy roots turned brown and

Table 1—Effect of age of *W. somnifera* plants on accumulation of withaferin A and withanolide A
[Values are mean ± SD from 10 observations each; ND = Not detected]

Age of the plant (days)	Withaferin A (µg/g dry wt.)		
	Root	Shoot	Leaf
120	ND	ND	673 (±43.3)
180	23 (±3.3)	16 (±3.3)	443 (±20.8)
210	136 (±5.7)	23 (±3.3)	1136 (±17.6)
	Withanolide A (µg/g dry wt.)		
120	136 (±12.0)	ND	823 (±20.2)
180	440 (±18.5)	ND	593 (±14.5)
210	13 (±3.3)	290 (±17.3)	586 (±17.6)

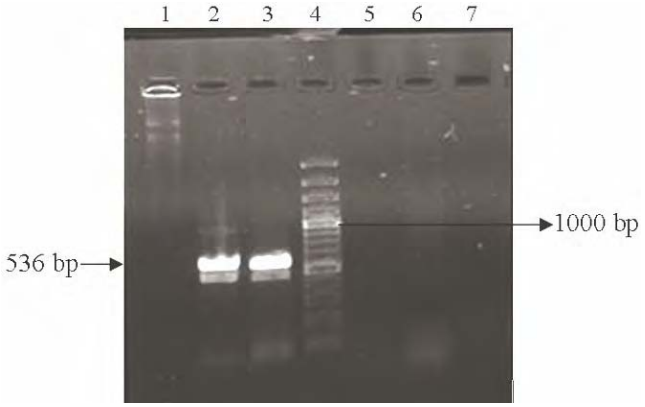


Fig. 2—PCR amplification of *rol*B gene in the strain 15834 and in hairy roots. [Lane 1= plasmid DNA isolated from *Agrobacterium rhizogenes* strain 15834. Lane 2= amplification *rol*B gene in strain 15834 (536 bp). Lane 3= amplification of *rol*B in hairy roots infected by the strain 15834 (536 bp). Lane 4= 1 kb ladder. Lanes 5, 6, 7= no amplification of *rol*B was observed in untransformed root, stem, leaf]

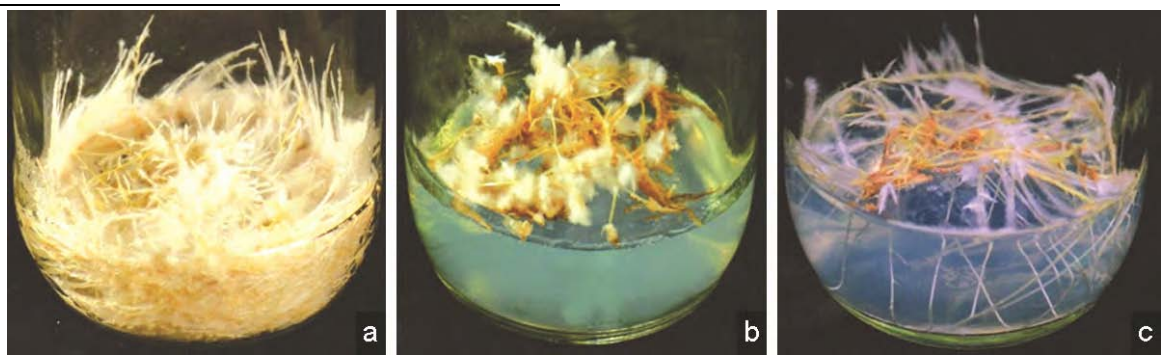
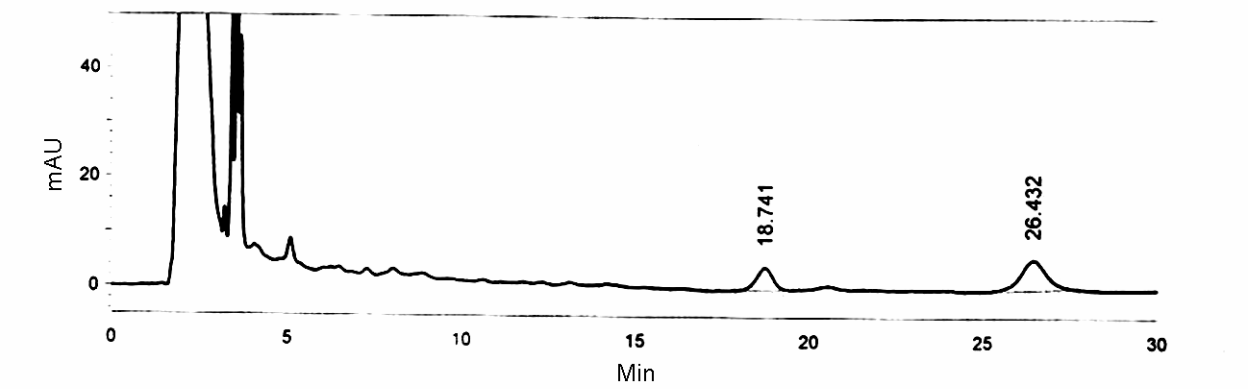


Fig. 1—Morphological variations observed in hairy root cultures of *W. somnifera* grown on MS basal medium containing 3% sucrose (Fig. 1a), 2% glucose (Fig. 1b) and 2% maltose (Fig. 1c).

necrosed. Increasing concentrations of glucose similarly increased the fresh and dry weights of hairy roots until 5% level. At 6 and 8% levels, growth of roots declined. Upon increasing the glucose from 1%, 696 and 2100 µg of withaferin A were observed respectively at 2 and 4% levels. However, no withanolide A was detected in these cultures. Accumulation of withaferin A was high (3866 µg)



1: 215 nm, 4 nm

Pk =	Retention Time	Area	Area Percent
1	18.741	141777	32.89
2	26.432	289295	67.11
Totals		431072	100.00

Fig. 3—HPLC chromatogram for withaferin A and withanolide A, isolated from hairy roots grown on MS medium containing 3% sucrose. Peaks indicate retention time for withaferin A (18.741) and withanolide A (26.432).

Table 2—Effect of different concentrations of carbohydrates on withaferin A and withanolide A accumulation in hairy root cultures of *W. somnifera* grown in MS basal medium
[Values are mean ± SD from 10 observations each]

Source of carbohydrate	Fresh weight g/culture	Dry weight g/culture	Withaferin A (µg/g dry wt)	% Increase in withaferin A	Withanolide A (µg/g dry wt)
Sucrose 1%	1.09 (±0.06)	0.08 (±0.01)	ND	-	ND
2% (control)	5.63 (±0.01)	0.27 (±0.04)	256 (±12)	-	ND
3%	5.92 (±0.03)	0.50 (±0.01)	890 (±11.5)*	347.6	886 (±25.9)
4%	20.02 (±0.33)	1.81 (±0.02)	1733 (±24)*	676.9	ND
6%	Tissues turned brown and died	-	-	-	-
Glucose 1%	2.41 (±0.67)	0.59 (±0.04)	ND	-	ND
2%	7.52 (±0.25)	0.49 (±0.04)	696 (±49.7)*	271.8	ND
4%	9.34 (±0.16)	1.94 (±0.05)	2100 (±20.8)*	820.3	ND
5%	15.12 (±0.32)	3.50 (±0.07)	3866 (±27.2)*	1510.1	350 (±18.5)
6%	10.97 (±0.49)	1.20 (±0.03)	410 (±23)*	160.1	ND
8%	2.82 (±0.20)	0.66 (±0.06)	923 (±20.2)*	360.5	ND
Fructose 1%	1.21 (±0.14)	0.19 (±0.03)	ND	-	ND
2%	1.55 (±0.15)	0.24 (±0.04)	ND	-	ND
3%	3.36 (±0.15)	0.56 (±0.04)	370 (±21.3)*	144.5	ND
4%	Tissues turned brown and necrosed	-	-	-	-

ND = Not detected; Values are significant at $^{*}P<0.05$ against control; % Increase in withanolide A is not represented since it is observed only in two treatments

when 5% glucose was incorporated into the medium. This medium also supported albeit to a smaller extent the accumulation of withanolide A (350 µg) unlike at other concentrations. Only withaferin A was detected (410 and 923 µg) at 6 and 8% glucose levels (Table 2). Fructose at 3% level stimulated the accumulation of withaferin A (370 µg/g DW), but did not support biomass and withanolide A (Table 2). Maltose and lactose were also added independently to the MS medium, but they did not support growth of hairy roots.

Effect of elicitors on withanolide accumulation in hairy roots—Withaferin A accumulation was higher in light (276 µg/g DW) compared to dark grown hairy roots (30 µg/g DW). Chitosan (100, 150 and 250 mg/L), JA (50, 100 and 150 mg/L) and ASA (0.5, 1.0 and 2.0 mg/L) were used in MS medium along with 2% sucrose. MS basal medium containing 2% sucrose served as the control. Increasing concentrations of chitosan slightly enhanced the growth of roots. Though withaferin A increased from 256 to 2160 µg/g DW at 150 mg/L, with further increase in chitosan (250 mg/L), accumulation of withaferin A declined to 1410 µg (Table 3). Higher concentrations of JA (150 mg/L) suppressed the growth of hairy roots by 2 to 2.5-folds. On the other hand, ASA stimulated more accumulation of withaferin A (1171 µg/g) compared to withanolide A

Table 3—Effect of elicitors, nitric oxide and triadimefon on the accumulation of withanolides in hairy root cultures of *W. somnifera* grown in MS basal medium
[Values are mean ± SD from 10 observations each]

Elicitor	Fresh weight g/culture	Dry weight g/culture	Withaferin A (µg/g DW)	% Increase /decrease over control	Withanolide A (µg/g DW)
MS + 2% sucrose (control)	5.63 (±0.01)	0.27 (±0.01)	256 (±12)	-	ND
MS + 100 mg/L chitosan	7.34 (±0.13)	0.59 (±0.01)	2000 (±20)*	781.2	ND
MS + 150 mg/L chitosan	8.37 (±0.02)	0.68 (±0.01)	2160 (±21)*	843.7	ND
MS + 250 mg/L chitosan	6.43 (±0.11)	0.69 (±0.03)	1410 (±12)*	550.7	ND
MS + 50 mg/L JA	5.16 (±0.34)	0.54 (±0.02)	240 (±10)	6.3 (decrease)	ND
MS + 100 mg/L JA	4.00 (±0.25)	0.60 (±0.03)	160 (±12)*	38.5 (decrease)	ND
MS + 150 mg/L JA	1.56 (±0.03)	0.26 (±0.02)	ND (tissues died)	-	ND (tissues necrosed)
MS + 0.5 mg/L ASA	6.75 (±0.18)	0.44 (±0.04)	2310 (±22)*	1015.6	ND
MS + 1 mg/L ASA	7.78 (±0.39)	0.65 (±0.08)	2690 (±32)*	1093.7	290
MS + 2 mg/L ASA	7.96 (±0.41)	0.72 (±0.09)	3060 (±35)*	1171.8	310
MS + 3 mg/L sodium nitroprusside	5.24 (+0.37)	0.54 (+0.1)	1274 (+29)	501.5	ND
MS + 30 mg/L sodium nitroprusside	5.98 (+0.46)	0.62 (+0.15)	1580 (+33)	617.1	ND
MS + 10 mg/L triadimefon	15.29 (±0.62)	0.79 (±0.11)	1873 (±30)*	731.6	ND
MS + 50 mg/L triadimefon	9.76 (±0.87)	0.70 (±08)	3137 (±26)*	1225.4	ND
MS + 100 mg/L triadimefon	5.01 (±0.26)	0.66 (±06)	4163 (±19)*	1626.2	ND

ND = Not detected; Values are significant at $P<0.05$ against control; % Increase in withanolide A is not represented since it is observed only in two treatments; JA = jasmonic acid; ASA = acetyl salicylic acid

(310 µg/g). Nitric oxide as released by sodium nitroprusside enhanced the accumulation of withaferin A to 1580 µg, while it did not affect withanolide A. Increasing concentrations of triadimefon, a fungicide, increased withaferin A in a dose dependent manner, the highest amount being 4163 µg/g DW. The increase in withaferin A content at 100 mg/L triadimefon was 1626 and 3061% (Table 3), compared to hairy roots grown on MS basal medium containing 2% sucrose and 210-day-old intact roots respectively, and this is the first report on such high accumulations in hairy roots of *Withania*.

Discussion

Existing evidence points that withanolides accumulate in roots by transportation from leaves[9]. Present study revealed that, irrespective of the age of plant, leaves of *W. somnifera* contained more withaferin A and withanolide A compared to roots. This lends support to the view that withanolides may be primarily accumulated in leaves. Contrary to this concept, Sangwan *et al.*[10] reported that withanolide A is inherently *de novo* biosynthesized in roots of *W. somnifera*. Further, the work of Chaurasiya *et al.*[1] and Sangwan *et al.*[11] elucidated that withanolides are biosynthesized in both leaves and shoots. This indicates that there may be redundant pathways for the biosynthesis of withanolides in *W. somnifera*.

Murthy *et al.*[12] reported low amounts of withanolide A (157.4 µg/g dry weight) in hairy roots at 4% sucrose level. On the other hand, 3% sucrose is the only carbohydrate where fair amount of withanolide A (886 µg/g DW) was also detected along with withaferin A in the present study. This is the first detection of withanolide A in such high concentrations in hairy root lines of *Withania*. This could be because of the differences in the selected hairy root lines. Several studies reported faster growth rates of transformed roots at higher sucrose concentrations than their non-transformed controls, for example, *Atropa belladonna*[13] and *Datura stramonium*[14]. Komaraiah *et al.*[15] reported the influence of glucose and fructose on increased biomass and plumbagin production in callus cultures of *Plumbago rosea*. Production of withaferin A was high when glucose was incorporated, while accumulation of withanolide A was hardly detected (Table 2). Both biomass (3.4978 g/culture on DW basis) and withaferin A (3866 µg) were optimum at 5% glucose level. Even withanolide A (350 µg) was detected at this glucose concentration. There appears to be a tilt for withaferin A accumulation rather than withanolide A in the hairy root line selected at higher sucrose and glucose concentrations. Whenever withaferin A content increased in the tissues, there was a concomitant decrease in withanolide A and vice versa (Tables 1-3). *W. somnifera* contains not only withanolides but also several other glycosides. Sterol glycosyltransferases catalyze the synthesis of diverse glycosteroids in plants. This leads to a change in their participation in cellular metabolism[16]. Since withanolides are glucoconjugates, glucose molecules provided in the medium may be utilized for glycosylation purpose. Therefore, an increase in withanolides in hairy root cultures of *W. somnifera* is logical when the cultures were fed with glucose. In the present study, only withaferin A (370 µg) but not withanolide A could be detected at 3% fructose level. Ray and Jha[4] reported shooty teratomas which were able to synthesize withanolides like that of the parent plant. They reported higher withanolide synthesis (not withanolide A) in shooty teratomas (0.07-0.1% withaferin A and 0.085-0.025% withanolide D) than in non-transformed shoot cultures. Similarly, Bandopadhyay *et al.*[6] reported accumulation of only withaferin A in hairy roots, but both withaferin A and withanolide D in rooty callus lines and callus.

In suspensions of *Polygonum*, 72% higher indurubin accumulated when subjected to elicitation with chitosan for a period of 5 days[17]. In *Plumbago rosea*, chitosan was the most effective and the highest amount of plumbagin accumulation was noticed as compared to the other elicitors[18]. Growth of hairy roots increased in presence of 150 mg/L chitosan in the present study. But growth declined with the addition of JA. In suspension cultures of *Morinda citrifolia*, anthraquinone production was affected by many plant growth regulators and maximum anthraquinone yield was obtained with methyl jasmonate/jasmonic acid (16.74 mg/g DW) when compared to controls[19]. Similarly, methyl jasmonate incorporation into the medium enhanced the production of asiaticoside from hairy root cultures of *Centella asiatica*[20] and suspensions of *Capsicum frutescens*[21]. Perhaps, this signaling molecule may be playing a negative role in the biosynthetic pathway of withanolides. ASA induced somatic embryogenesis and also increased the accumulation of plumbagin in suspensions[18]. Salicylic acid altered the levels of hormones and subsequently growth in wheat[22].

Further, it is associated with defense response and hence stimulation of secondary product accumulation seems plausible. Diverse developmental and physiological processes in plants are associated with nitric oxide, being an important signal transducing molecule. In sweet potato, nitric oxide regulated the gene expression involved in the alkaloid biosynthesis[23]. Together with hydrogen peroxide, nitric oxide may regulate plant defenses and also the secondary metabolite synthesizing pathways[19]. The effect of triadimefon on secondary metabolite accumulation has not been studied earlier in callus or suspensions or hairy roots. Also, the mode of action or the mechanism by which it could trigger the accumulation of withferin A significantly is not known. These hairy root cultures were maintained for more than 20 months and the contents of both withaferin A and withanolide A did not decline. Thus, present study demonstrates the need for harnessing the potential of hairy root cultures of *W. somnifera* for large scale production of withanolides.

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